

Involvement of calcium, calmodulin and phospholipase A in the alteration of membrane dynamics and superoxide production of human neutrophils stimulated by phorbol myristate acetate

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We have reported an increased fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the phorbol myristate acetate-stimulated plasma membrane of human neutrophils [FEBS Lett. (1982) 144, 199–203]. We now present evidence that both the increased fluorescence polarization and the production of superoxide radicals by human neutrophils require calcium, calmodulin and phospholipase activity.

Membrane dynamics Order parameter Neutrophil Calcium Calmodulin
Phospholipase A

1. INTRODUCTION

Polymorphonuclear leucocytes (PMN), when activated by a variety of soluble or particulate stimuli, exhibit chemotaxis, aggregation, degranulation, phagocytosis and a dramatic increase in respiration due to activation of a membrane-associated NADPH oxidase [1]. Activation begins with the binding of the stimulus to specific plasma membrane-located receptor sites, followed by a transfer of signals across the plasma membrane that lead to the cellular responses.

Modification of membrane phospholipids might be important for transmembrane signalling [2,3] as well as for liberation of arachidonic acid, a precursor of the lipoxygenase and cyclooxygenase system, and a substrate for the superoxide-dependent production of chemotactic factors [4]. Indeed, a phospholipase A₂ activity has been demonstrated to occur in human PMN [5–7].

Little is known about the physical consequences

due to stimulus recognition and signal transfer in the plasma membrane. We have shown [8] that the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) increases in the phorbol myristate acetate (PMA)-stimulated plasma membrane in human PMN. This increase suggests an increased order of phospholipid fatty acid side chains upon stimulation with PMA. Here we present evidence that the increased fluorescence polarization r^s is due to the action of a phospholipase associated with the plasma membrane. It is likely that calcium and calmodulin act as second messengers for this enzyme.

2. MATERIALS AND METHODS

Chemicals were obtained from the following sources: Fluka (Buchs), 4-bromo-phenacyl-bromide; Sigma (St Louis), EGTA, phospholipase C from *C. perfringens*; Boehringer (Mannheim), phospholipase A₂ from hog pancreas; University Hospital (Zürich), nupercaine; trifluoperazine was a gift of Smith, Kline and French (Philadelphia). All other chemicals and methods were as in [8].

3. RESULTS AND DISCUSSION

PMA causes a rapid and lasting increase in the

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Abbreviations: DPH, 1,6-diphenyl-1,3,5,-hexatriene; EGTA, ethylene glycol-bis (β -amino ethylether)-*N,N'*-tetraacetic acid; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear leucocytes

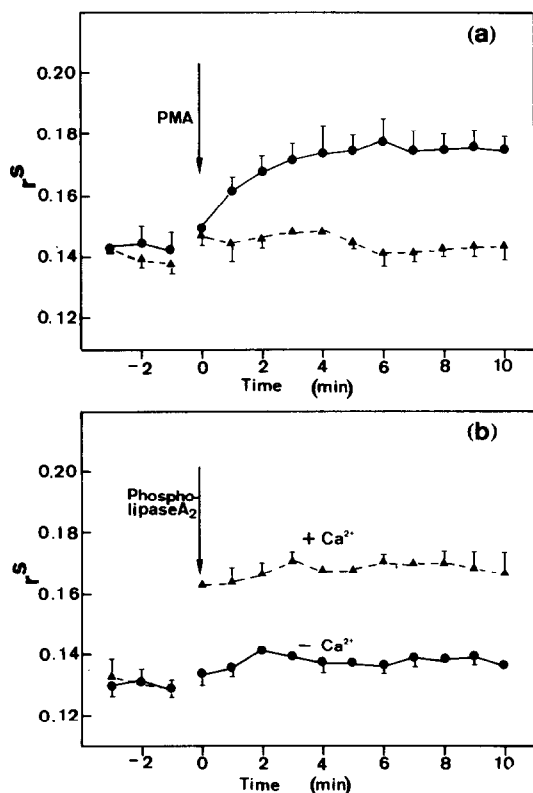


Fig.1. Steady-state fluorescence anisotropy, r^s , of DPH in the plasma membrane fraction of PMN. Cells were labeled with DPH as in [8]. (a) Stimulation (\rightarrow) by 1 μ g PMA/ml; (—), control; (---) in the presence of 10 μ M trifluoperazine, added 10 min before PMA. (b) In the presence of 7 units phospholipase A_2 (from hog pancreas, \rightarrow); (—), no Ca^{2+} added; (---), in the presence of 1 mM Ca^{2+} . The results represent the mean value of 2 (a) or 3 (b) separate experiments (\pm SD), each performed in duplicate.

fluorescence polarization value r^s of DPH in the plasma membrane of human neutrophils [8]. This finding is confirmed in fig.1a. However, the molecular events leading to this increase of r^s are not yet known. The increase can be prevented by 20 μ M trifluoperazine (fig.1a), a potent inhibitor of a variety of calmodulin-sensitive enzymes [9], among them phospholipase A_2 [10,11]. Indeed added phospholipase A_2 (7 units) can mimic PMA in causing a rapid and lasting increase of r^s (fig.1b). The exogenous phospholipase is strongly stimulated by added calcium (fig.1b). Addition of phospholipase C (7 units) to the isolated mem-

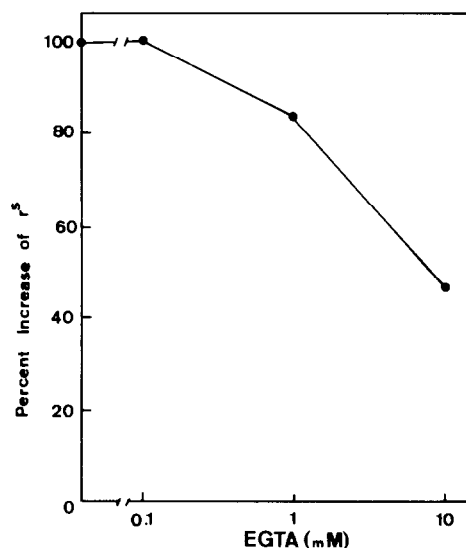


Fig.2. Inhibition by EGTA of the PMA induced increase of r^s . Experimental conditions as in fig.1a. r^s was determined 6 min after the addition of PMA. 100% refers to the r^s value obtained in the absence of EGTA.

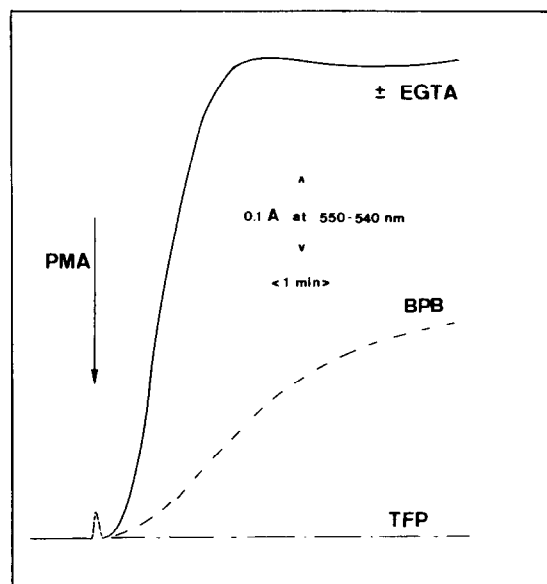


Fig.3. Superoxide radical-dependent reduction of ferricytochrome c by PMN, stimulated by 1 μ g PMA/ml: (—) control \pm 10 mM EGTA; (---) in the presence of 10 μ M 4-bromophenacyl-bromide; (- · -) in the presence of 10 μ M trifluoperazine.

brane fraction in the presence or absence of calcium did not change r^s .

Although the isolation of the plasma membrane fraction and fluorescence polarization measurements were performed in Hank's buffered salt solution not supplemented with calcium also the PMA induced increase of r^s appears to be dependent on calcium, since EGTA diminishes the extent of the increase in a dose-dependent fashion (fig.2).

The existence of a membrane-bound phospholipase A_2 [7] can easily explain the r^s increase and its continuance upon PMA stimulation. It is known that the order of the lipid bilayer is dependent on the degree of saturation of fatty acid side chains, and that predominantly the highly unsaturated arachidonic acid is hydrolyzed from membrane phospholipids upon activation of PMN [5]. Liberation of arachidonic acid by phospholipase

A_2 , and not by the combined action of phospholipase C and diacyl glycerol lipase, has been suggested [5]. The increase of r^s in the presence of added phospholipase A_2 is in line with the above findings. While the exogenous phospholipase A_2 must be furnished with calcium the membrane-bound phospholipase apparently has a high affinity for calcium because mM levels EGTA are required to inhibit substantially the PMA-induced r^s increase. The importance for PMN stimulation of calcium tightly associated with the plasma membrane was already suggested in [12].

4-Bromophenacyl-bromide (10 μ M) and nupercaine (10 μ M), inhibitors of phospholipase A [13,14] interfered with the fluorescent properties of DPH and could therefore not be used as probes for the phospholipase-dependent change of r^s .

The causal and temporal relationship between the r^s increase of DPH and the production of su-

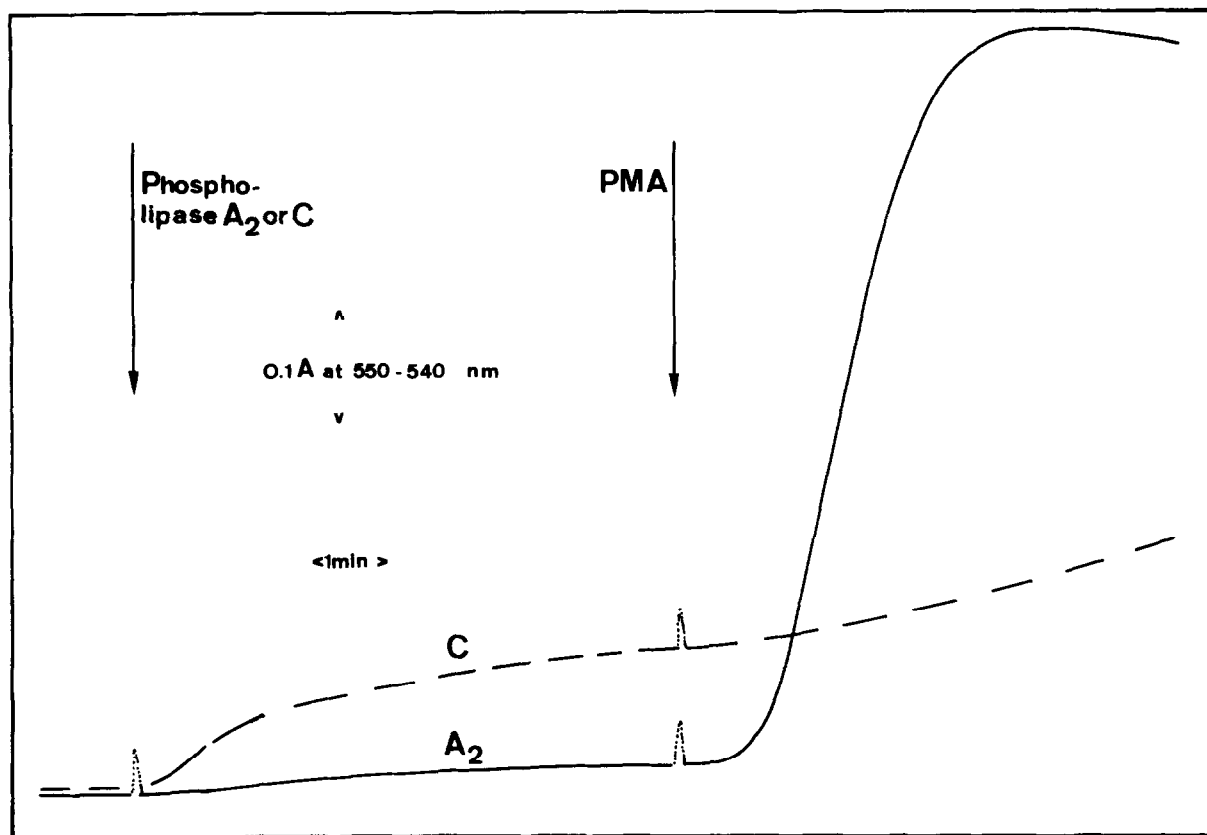


Fig.4. Superoxide radical-dependent reduction of ferricytochrome *c* by PMN challenged with 7 units of phospholipase A_2 (—) or 7 units phospholipase C (---) (first arrow). The second arrow shows the addition of 1 μ g PMA/ml.

peroxide radicals ($O_2^{\cdot-}$) is not yet clear. EGTA, trifluoperazine, phospholipases and their inhibitors were therefore tested in the above concentrations for the ability to modulate $O_2^{\cdot-}$ production in isolated human neutrophils. Fig.3 shows that the $O_2^{\cdot-}$ production is completely inhibited by 20 μ M trifluoperazine, and largely (80%) by 10 μ M 4-bromophenacyl-bromide while EGTA up to 10 mM was without effect.

The lack of inhibition of $O_2^{\cdot-}$ production in PMN by EGTA shown here supports the conclusion that external calcium is not required for the stimulation of superoxide production [15]. Rather, both increased r^s and superoxide production seem to be dependent on intracellular calcium and on calmodulin as indicated by trifluoperazine inhibition. Chlorpromazine, a weaker calmodulin antagonist, inhibits $O_2^{\cdot-}$ production in human PMN [15].

When phospholipase A₂ or C instead of PMA were added to human neutrophils (fig.4) the rate as well as the extent of $O_2^{\cdot-}$ production were 5% and 20%, respectively, of that observed after addition of PMA. PMA added subsequent to phospholipase A₂ resulted in a normal burst of $O_2^{\cdot-}$ production. Phospholipase C largely prevented further stimulation by PMA (fig.4). The substrate specificity and the site of action of the plasma membrane-associated phospholipase indicated here is not known. The reduced $O_2^{\cdot-}$ production in the presence of added phospholipase A₂ or C could therefore be due to topological constraints or different substrate affinity of the endogenous phospholipase. In any case, the sensitivity towards PMA stimulation is preserved after addition of phospholipase A₂. However, this is not the case for phospholipase C. The activity of this enzyme can be expected to result in alterations of the charge distribution at the plasma membrane which might prevent subsequent binding of PMA to the cells.

Taken together, the results suggest the participation of calcium, calmodulin and a plasma membrane-associated phospholipase in the alteration of membrane dynamics and superoxide production in the stimulation of human neutrophils. It is tempting to speculate that upon stimulation of PMN a sequential interaction between calcium, calmodulin and phospholipase takes place. Whether the

increased r^s is only due to liberation of unsaturated fatty acids by an activated phospholipase, or also by altered protein-protein interactions possibly due to the changed lipid environment requires further investigation.

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